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DEXAMETHASONE SUPPRESSES THE EXPRESSION OF MULTIPLE RAT CARBOXYLESTERASES THROUGH TRANSCRIPTIONAL REPRESSION: EVIDENCE FOR AN INVOLVEMENT OF THE GLUCOCORTICOID RECEPTOR

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Abstract

Carboxylesterases play important roles in the metabolism of xenobiotics and detoxication of insecticides. Without exception, all mammalian species studied express multiple forms of carboxylesterases. Several rat carboxylesterases are well-characterized including hydrolase A, B and S, and the expression of these enzymes is significantly suppressed by glucocorticoid dexamethasone. In this study, we used multiple experimental systems and presented a molecular mechanism for the suppression. Rats receiving one or more daily injections of dexamethasone consistently expressed lower HA, HB and HS. The suppression occurred at the levels of mRNA, protein and hydrolytic activity. In hepatoma cell line H4-II-E-C3, nanomolar dexamethasone caused significant decreases in HA, HB and HS mRNA, and the decreases were abolished by antiglucocorticoid RU486. Additionally, dexamethasone at nanomolar concentrations repressed the promoters of carboxylesterases, and the repression was reduced by glucocorticoid receptor- β , a dominant negative regulator of the glucocorticoid receptor (GR). In contrast, co-transfection of the pregnane X receptor (PXR) increased the reporter activities, but the increase occurred only at micromolar concentrations of dexamethasone. These findings establish that both GR and PXR are involved in the regulated expression of rat carboxylesterases by dexamethasone but their involvement depends on the concentrations.

INTRODUCTION

Carboxylesterases play an important role in the metabolism of endogenous lipids and foreign compounds containing such functional groups as carboxylic acid ester, amide and thioester (Satoh and Hosokawa, 2006; Shi et al., 2006). In addition to hydrolysis, some carboxylesterases catalyze transesterification reaction, which accounts for the conversion of anti-platelet agent clopidogrel (a methyl ester) to ethyl clopidogrel (the corresponding ethyl ester) (Tang et al., 2006). While carboxylesterase activity is widely distributed in mammalian tissues, the highest level is present in liver microsomes (Satoh and Hosokawa, 2006). The abundant presence of carboxylesterases in the liver is linked to certain cellular structural roles, particularly in directing protein trafficking (Ovnic et al., 1991). For example, egasyn, a liver microsomal

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carboxylesterase, binds to β -glucuronidase via its active site, and such binding results in sequestration of this enzyme within the endoplasmic reticulum (Ovnic et al., 1991). Organophosphorus insecticides target the active site and release easily-complexed β -glucuronidase into the blood (Satoh et al., 1999). Organo-phosphorus compounds such as fenitrothion at nanomolar concentrations cause significant increases of β -glucuronidase in the blood, thus serving as a sensitive biomarker for the exposure to these insecticides (Satoh et al., 1999).

Mammalian species express multiple forms of carboxylesterases (Satoh and Hosokawa, 2006). The well characterized examples include rat hydrolase A, B and S (HA, HB, HS) (Yan et al., 1995a; Yan et al., 1995b; Yan et al., 1994; Morgan et al., 1994), and human carboxylesterase HCE1 and HCE2 (Schwer et al., 1997; Kroetz et al., 1993). These carboxylesterases generally have a sequence identity of ~70% with an exception of HCE2, which shows ~50% identity with other carboxylesterases (Satoh and Hosokawa, 2006). Like many other xenobiotic-metabolizing enzymes (Yang et al., 2008; Choudhary et al., 2004), the expression of carboxylesterases is regulated developmentally as well as by many xenobiotics. Based on Western analysis, neither HA nor HB is expressed in 3-week old or younger rats (Morgan et al., 1994). Both carboxylesterases are induced by phenobarbital and clofibrate, however, the induction is only minimal (15 and 30%) (Yan et al., 1995b; Morgan et al., 1994). Based on immunoprecipitation study, hydrolase A and B together contribute 90% of the hydrolytic activity toward *p*-nitrophenylacetate (Morgan et al., 1994).

In contrast to the induction, suppression of rat carboxylesterases is profound by several chemicals (Poole et al., 2001; Yan et al., 1995b; Morgan et al., 1994). For example, treatment of mature rats with dexamethasone causes as much as 80% decrease in hydrolytic activity toward *para*-nitrophenylacetate (a standard carboxylesterase substrate), the corresponding proteins and mRNAs of HA, HB and HS (Zhu et al., 2000; Yan et al., 1995b; Morgan et al., 1994). The suppression, however, does not occur with pregnenolone-16 α -carbonitrile, an antiglucocorticoid (Yan et al., 1995b). In cultured rat hepatocytes, dexamethasone caused significant suppression even at nanomolar concentrations (Zhu et al., 2000). Two major receptors are known to mediate the action of dexamethasone: the glucocorticoid receptor (GR) (Pei, 1996) and the pregnane X receptor (PXR) (Kliwer et al., 1998). The activation of GR requires nanomolar, whereas the activation of PXR requires micromolar concentrations of dexamethasone. In addition, GR is activated by glucocorticoids only, but PXR can be activated by both glucocorticoids and anti-glucocorticoids.

The aim of this study was to test the hypothesis that dexamethasone suppresses the expression of rat carboxylesterases through GR. Rats and hepatoma cell line were treated with dexamethasone at various doses or concentrations. As expected, dexamethasone caused significant and persistent suppression of HA, HB and HS in rats and hepatoma cell line. The suppression, however, was abolished by antiglucocorticoid RU486, but not by protein synthesis inhibitor cycloheximide, suggesting that the suppression is achieved through GR-mediated trans-repression and does not require on-going protein synthesis. In addition, dexamethasone repressed the promoters of rat carboxylesterase, and the repression was reduced by glucocorticoid receptor- β , a dominant negative regulator of glucocorticoid receptor- α . Interestingly, the promoter sequences supporting the repression lack canonical glucocorticoid response elements. These findings establish that glucocorticoid receptor- α is involved in the suppression of rat carboxylesterases but through a nonclassic mechanism.

MATERIALS AND METHODS

Chemicals and supplies

Cortisol, cycloheximide, dexamethasone, equine serum, *para*-nitrophenylacetate, Hanks balanced salt solution and RU486 were purchased from Sigma (St. Louis, MO). Minimum essential medium eagle (MEME) and high fidelity Platinum *Taq* DNA polymerase were purchased from Invitrogen (Carlsbad, CA). Reporter Assay System was from Promega (Madison, WI). Fetal bovine serum was from HyClone laboratories (Logan, UT). Ketamine HCl was purchased from Fort Dodge Animal Health (Fort Dodge, IA). The antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Abcam (Cambridge, UK). The goat anti-rabbit IgG conjugated with horseradish peroxidase was from Pierce (Rockford, IL). Nitrocellulose membranes were from Bio-Rad (Hercules, CA). Unless otherwise specified, all other reagents were purchased from Fisher Scientific (Fair Lawn, NJ).

Animal treatment

Sprague-Dawley male rats from Charles River (Wilmington, MA) were injected *ip* once daily with dexamethasone (50 mg/kg) in corn oil or the same volume of vehicle (Zhang et al., 1999). At a given time-point for sacrifice, rats were *ip* injected with ketamine (1 ml/kg at 100 mg/ml). After rats were completely anesthetized (~10 min), surgery was performed to expose the liver. The liver was perfused with phosphate buffered saline (37°C) through the portal vein to remove blood. The perfused liver was then divided into two parts. One part was immediately used for preparing total RNA and the remaining part was frozen at -80°C for preparing S9 fractions. All rats were allowed free access to Purina Rodent Chow 5001 and water, and the use of animals was approved by the Institutional Animal Care and Use Committee.

Cell culture and treatment

Rat hepatoma line H4-II-E-C3 was purchased from the American Type Culture Collection (Rockville, MD). The hepatoma cells were maintained in MEME containing 10% fetal bovine serum, 5% equine serum, penicillin (100 units per ml)/streptomycin (100 µg/ml), 1x non-essential amino acids and 1 mM sodium pyruvate. Cells were usually seeded at a density of 5×10^5 cells/well (12-well plates) in normal medium. After an overnight incubation, treatment was started with dexamethasone or the same volume of DMSO. In some cases, repeated treatment was performed 24 h after the initial treatment with fresh medium containing dexamethasone at the same concentration. The duration and concentration of treatment are specified in figure legends.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated with an RNA-Bee (Friendswood, TX) according to the manufacturer's manual, and the integrity of the RNA was confirmed by formaldehyde gel electrophoresis. Total RNA (1 µg) was subjected to the synthesis of the first strand cDNA in a total volume of 25 µl with random primers and M-MLV reverse transcriptase. The reactions were conducted at 25°C for 10 min, 42°C for 50 min and 70°C for 10 min. The cDNAs were then diluted 8 times and quantitative PCR was conducted with *TaqMan* Gene Expression Assay (Applied Biosystems, Foster City, CA). The *TaqMan* assay identification numbers were: HA, Rn00591030_m1; HB, Rn01774462_m1; HS, Rn00592205_m1; tyrosine aminotransferase (Tat), Rn00562011_m1; rat GAPDH, Rn99999916_s1; and rat polymerase II, Rn01752026_m1. The PCR amplification was conducted in a total volume of 20 µl containing universal PCR master mixture (10 µl), gene-specific *TaqMan* assay mixture (1 µl), and cDNA template (6 µl). Cycling profile was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60 °C, as recommended by the manufacturer. The mRNA levels were normalized according to the level of GAPDH and RNA polymerase II (selected samples)

(Radonic et al., 2004). Amplification and quantification were done with the Applied Biosystems 7900 Real-Time PCR System.

Preparation of S9 fractions and hydrolysis of *para*-nitrophenylacetate

Frozen livers (perfused) were thawed in homogenization buffer (50 mM Tris-HCl, pH 7.4, 150 mM KCl and 2 mM EDTA) and then homogenized with 6 passes of Teflon pestle driven by a Wharton stirrer. The homogenates were centrifuged at 10,000 *g* for 20 min at 4°C. The S9 fractions (supernatant) were assayed for the hydrolysis of *para*-nitrophenylacetate as described previously (Yang et al., 2007). Sample cuvette (1 ml) contained 20 µg S9 fractions in 100 mM potassium phosphate buffer (pH 7.4), and 1 mM substrate at room temperature. Reactions were initiated by adding *para*-nitrophenylacetate (10 µl of 100 mM stock in acetonitrile) and hydrolytic rate was recorded from an increase in absorbance at 400 nm. The extinction coefficient (E_{400}) was determined to be 13 mM⁻¹ cm⁻¹, and non-enzymatic hydrolysis was subtracted (Yang et al., 2007).

Reporter constructs

Luciferase reporters harboring rat carboxylesterase promoters at varying length (HB and HS only) were prepared by inserting the corresponding genomic fragment into the pGL4.10 vector. To prepare HB reporters, a genomic fragment from -3590 to -172 (relative to the translation initiation codon) was amplified by PCR with high fidelity Platinum *Taq* DNA polymerase and rat genomic DNA as the template. The fragment was generated with forward primer HB-3590XhoIs (5'-ccttatctcgagtcggtgaactg-acaatgttactat-3') and reverse primer HB-172BglIIa (5'-aagccagatctcttccaagtttacctggctttatct-3'). This fragment was digested with Xho I and Bgl II and ligated to the pGL4.10 vector pretreated with the same endonucleases. This reporter was designated as HB-3590-Luc. To prepare 5' truncated constructs of this reporter, the corresponding genomic DNA fragments were amplified by PCR with the HB-3590-Luc reporter as the template. These 5' deleted genomic fragments were amplified with the same reverse primer but different forward primers (Table I). Similarly, the PCR-amplified fragments were ligated to the pGL4 vector. To prepare the HB-4822-Luc reporter (containing the longest HB genomic sequence among all HB reporters), a further upstream genomic fragment was generated with forward primer (HB-4822KpnIs) and reverse primer (HB-3537NheIa). This fragment was digested with Kpn I and Nhe I (at -3610 to -3616) and ligated to the HB-3590-Luc pretreated with the same restriction endonucleases to produce the HB-4822-Luc reporter. This reporter had a 20-bp deletion (-3610 to 3590) and 8-bp insertion of the vector sequence. The reporters for hydrolase S (HS) were prepared similarly. The HS-1293-Luc reporter was constructed first and used as the template for preparing 5' truncated reporters. The sequences of primers are shown in Table I. All reporters were subjected to sequence analysis.

Co-transfection assays

Hepatoma cells (H4-II-E-C4) were plated in 48-well plates in MEME supplemented with 10% delipided fetal bovine serum at a density of 1.2×10^5 cells per well. Transfection was conducted by FuGene HD (Roche, Indianapolis, IN). Transfection mixtures contained 100 ng of a reporter plasmid and 10 ng of TK-*Renilla* luciferase plasmid. Cells were transfected for 8 h and the medium was replaced with fresh medium without serum. After cultured for 24 h, the transfected cells were treated with dexamethasone for 48 h with a change of the medium at 24 h (dexamethasone kept the same). The cells were washed once with phosphate buffered saline, and harvested in passive lysis buffer. After 30-min incubation at room temperature with shaking, the reporter activities were assayed with a Dual-Luciferase Reporter Assay System for the activities of two luciferases. The firefly luciferase activity, which represented the reporter activity, was initiated by mixing an aliquot of lysates (10 µl) with Luciferase Assay Reagent II. Then the firefly luminescence was quenched and the *Renilla* luminescence was

simultaneously activated by adding Stop & Glo Reagent to the sample tubes. The firefly luminescence signal was normalized based on the *Renilla* luminescence signal. To specify an involvement of the glucocorticoid receptor or the pregnane X receptor (PXR), a glucocorticoid receptor- β , PXR construct or the same amount of vector (100 ng) was added to the transfection mixture. The glucocorticoid receptor- β expression construct was kindly provided by Dr. Yorio of the University of North Texas Health Science Center (Zhang et al., 2005), and the PXR construct was described elsewhere (Zhang et al., 1999).

Western analysis

Liver homogenates (4–8 μ g) were resolved by 7.5% SDS-PAGE in a mini-gel apparatus and transferred electrophoretically to nitrocellulose membranes. After non-specific binding sites were blocked with 5 % non-fat milk, the blots were incubated with an antibody against HA, HB or GAPDH (Zhu et al., 2000). The primary antibodies were subsequently localized with goat anti-rabbit IgG conjugated with horseradish peroxidase. Horseradish peroxidase activity was detected with a chemiluminescent kit (SuperSignal West Pico). The chemiluminescent signal was captured by KODAK Image Station 2000 and the relative intensities were quantified by KODAK 1D Image Analysis Software.

Other analyses Protein concentrations were determined with BCA assay (Pierce) based on bovine serum albumin standard. Data are presented as mean \pm SD of at least three separate experiments, except where results of blots are shown in which case a representative experiment is depicted in the figures. Statistical analysis was performed using SAS software (SAS®, version 9.1, Cary, NC, USA). Significant differences were made according to One-way ANOVA followed by a DUNCAN's multiple comparison test ($p \leq 0.05$). Data points (or bars) with statistically significant differences are assigned a different letter (sharing no letter) or labeled by an asterisk sign. Location of DNA elements was performed with the Genomatrix program.

RESULTS

Dexamethasone persistently decreases the expression of carboxylesterases in rats

The effect of dexamethasone on the expression of rat carboxylesterases has been studied by several investigators (Zhu et al., 2000; Crunkhorn et al., 2004; Furihata et al., 2005). In these studies, rats were treated by multiple *ip* injections (3–4 consecutive days with a daily injection). The dose varied from 5 to 100 mg/kg body weight, and the method to monitor the expression varied as well. These variables likely contributed to inconsistent reports with some showing induction but others suppression (Zhu et al., 2000; Crunkhorn et al., 2004; Furihata et al., 2005). In this study, we adopted a protocol in which rats received a maximum of four consecutive daily injections (50 mg/kg/day) and the livers were harvested at multiple time-points after the initial treatment (6, 12, 24, 48, 72 or 96 h). The protocol was designed to gain information on transient and accumulated effect (single versus repeated injections). The levels of mRNA, protein and enzymatic activity were monitored to shed light on the mechanism of action involved in the regulated expression of carboxylesterases. In addition, the expression of Tat was monitored as positive control for transactivation (Becker et al., 1986).

The results on the animal study are summarized in Fig. 1. The level of Tat mRNA was rapidly increased by as many as 6 fold during the first 6-h treatment (Fig. 1A). Interestingly, the increase was reduced with prolonged treatment. At the 12-h time-point, the increase was reduced to an approximately 3-fold; at the 24-h time-point, the increase was minimal (~30%); and at the 96-h time-point, no increase was detected (Fig. 1A). In contrast, the mRNA levels for HA, HB and HS were persistently decreased in the livers collected at all time-points (Figs. 1B–1D). The level of HA mRNA was decreased by 40% at the 6-h time-point and reached the

maximum (~70%) at the 48-h time-point (Fig. 1B). The level of HB mRNA was decreased by 30% at the 6-h time-point and 65% at the 48-h time-point (Fig. 1C). The level of HS mRNA was decreased by 50% at the 6-h time-point and 85% at the 48-h time-point (Fig. 1D). Prolonged treatment (72 and 96 h) caused some changes in the mRNA levels of carboxylesterases. These changes were statistically significant from controls and at the 6-h time-point, but not the 24-h time-point (bars labeled with the same letter). Overall, the magnitude of the decreases was greater on HS than HA and HB based on respective mRNA levels. Control rats receiving corn oil for each time-point showed little changes on the level of mRNA for all genes monitored (data not shown).

We next examined whether the decreases of HA, HB and HS mRNA translate into decreases in the hepatic hydrolysis. Livers from dexamethasone-treated rats were perfused and S9 fractions were prepared. The hydrolytic activity of the S9 fractions was determined with *para*-nitrophenylacetate, a colorimetric substrate of HA, HB and HS (Yan et al., 1995b). As shown in Fig. 1E, the overall hydrolytic activity was decreased, and the decrease was greater in the samples from rats receiving longer treatment. The maximum decrease was 45% and detected in the samples collected at the 96-h time-point (Fig. 1E). Next, we examined whether the decreased activity is correlated with the levels of HA and HB. HS is a secretory carboxylesterase and contributes little to the hepatic hydrolysis (Yan et al., 1995b). As shown in Fig. 1E (Western blots), no decrease was detected on either HA or HB in the samples collected at the earlier time-points (e.g., 6-, 12-h), whereas a 30–80% decrease was detected in the samples collected at later time-points (Fig. 1E). According to GAPDH-normalized immunostaining intensities, HA was decreased by 34% at the 72-h time point and by 56% at the 96-h time point, and HB was decreased by 30, 60 and 80% at the 48-, 72- and 96-h time points, respectively. It was evident that the change on mRNA preceded the change on the corresponding protein (Figs. 1B, C and E).

The carboxylesterase genes are sensitive targets of dexamethasone

The animal study demonstrated that dexamethasone significantly induced the expression of Tat but suppressed HA, HB and HS. The induction of Tat was transient whereas the suppression was sustained over the entire duration of the observation (Figs. 1A–D). We next examined whether such a differential response could be recaptured in cell lines. Hepatoma cells (H4-II-E-C3) were treated with dexamethasone ranging from 1 nM to 10 μ M for 6, 12, 24 and 48 h. The medium was replaced with fresh medium at 24 h containing the same concentration of dexamethasone. Similarly, RT-qPCR was used to monitor the expression. The mRNA levels were normalized and expressed as the relative level (dexamethasone-treated cells over the corresponding controls).

The results on the cell line study are summarized in Fig. 2. The levels of Tat mRNA were increased in both 6- and 24-h cultures. The maximum increase in the 6-h cultures was ~8 fold, and ~30 fold in the 24-h cultures (Fig. 2A). In the 6-h cultures, only relatively high concentrations (>50 nM) caused statistically significant increases, whereas in the 24-h cultures, significant increases were detected with all concentrations except 1 nM (Fig. 2A). The changes in the levels of carboxylesterase mRNA varied depending on a gene and the length of treatment (Figs. 2B–D). In the 6-h cultures, little changes were detected in the level of HB mRNA (Fig. 2C), whereas the levels of HA and HS mRNAs showed some changes but depending on the concentrations. The level of HA mRNA showed little changes at low concentrations (50 nM or lower) but was significantly increased at higher concentrations (100 nM or higher) (Fig. 2B). HS mRNA, on the other hand, was decreased with all concentrations (Fig. 2D). In the 24-h cultures, the levels of all carboxylesterase mRNAs were profoundly decreased at low concentrations with HA mRNA being decreased to the least extent (Figs. 2B–D). At high concentrations, however, the decreases were much less. As a matter of fact, HA mRNA was

increased by as many as ~5 fold at 10 μ M. It should be noted that the changes (for both Tat and carboxylesterases) at the 12 h time-point were slightly more profound than those at the 6 h time-point, and the changes at the 48 h time-point were more than those at the 24 h time-point (data not shown). Cortisol, a natural glucocorticoid, caused a similar pattern of changes in Tat and carboxylesterase mRNA, but it was less potent than dexamethasone. The effect of cortisol on HB mRNA is shown in Fig. 2E.

RU486, but not cycloheximide, abolishes dexamethasone-mediated changes in HA, HB and HS mRNA

It is well established that induction of Tat by dexamethasone is achieved through transactivation directed by the glucocorticoid receptor. We next examined whether this receptor also supports the suppression of rat carboxylesterases. Hepatoma cells (H4-II-E-C3) were treated with dexamethasone in the presence or absence of RU486, a potent antagonist of the glucocorticoid receptor. As shown in Fig. 3A, this antiglucocorticoid alone caused little changes in the levels of carboxylesterase mRNAs. However, RU486 markedly or completely abolished dexamethasone-mediated decreases in HA, HB and HS mRNA (Fig. 3A). As expected, the induction of Tat by dexamethasone was completely abolished by this antiglucocorticoid (Fig. 3A). Next we tested whether the suppression requires on-going protein synthesis, the same cell line was treated with dexamethasone in the presence or absence of cycloheximide (CHX), a protein synthesis inhibitor. The mRNA level of α -1 acid glycoprotein (AGP) rather than Tat was monitored as a control, because induction of AGP but not Tat by dexamethasone requires on-going protein synthesis (Klein et al., 1988; Becker et al., 1986). As expected, dexamethasone alone increased AGP mRNA and decreased the mRNA levels of carboxylesterases. The increase in AGP mRNA, however, was completely abolished by CHX. In contrast, this protein synthesis inhibitor showed no effect on the decreases of carboxylesterase mRNAs.

Dexamethasone represses the promoter of HB and HS

The abolished suppression by RU486 suggests that dexamethasone down-regulates the expression through the glucocorticoid receptor. We next tested whether treatment with dexamethasone directly represses carboxylesterase promoters. To this end, we have prepared promoter reporters harboring HB or HS regulatory sequences with various lengths (Left of Figs. 4A and B). Among these reporters, the HB-4822-Luc reporter contained the longest promoter sequence with a putative binding site for the glucocorticoid receptor (Left of Fig. 4A). Preliminary studies showed that cells cultured in serum-free medium supported higher repression, therefore, the reporter assays were cultured in the absence of serum.

All HB reporters were significantly repressed by dexamethasone except HB-580-Luc and HB-559-Luc (Fig. 4A). The HB-4822-Luc reporter exhibited the maximum repression (~90%), and the others were repressed by 50–70%. The HB-580-Luc and HB-559-Luc reporters, on the other hand, showed little basal activity, suggesting that the genomic sequence from –620 to –559 is critical for both basal and dexamethasone-regulated transcription. Overall, the HS reporters were repressed by ~50% except the HS-282-Luc reporter, which was repressed by ~10% (Fig. 4B). The HS-282-Luc reporter contained the shortest genomic sequence. The reduced repression of this reporter, compared with the repression of the HS-630-Luc reporter, suggests that genomic sequence from –630 to –282 contributes significantly to the overall repression of the HS promoter. Several transcription factors such as activator protein-1 and nuclear factor- κ B are reported to involve the signaling of glucocorticoid (Dostert and Heinzel, 2004; Adcock and Caramori, 2001). However, inhibitors on these signaling pathways showed no effect on dexamethasone repression of rat carboxylesterases (data not shown).

Attenuated repression of HB and HS promoters by glucocorticoid receptor- β and PXR

The study with promoter reporters established that transcription repression is involved in the suppression by dexamethasone. In order to gain a quantitative-response insight, one HB and one HS reporter were tested for their responsiveness to increasing concentrations of dexamethasone ranging from 1 nM to 100 μ M. The selection of the range of the concentrations was based on the study with hepatoma line H4-II-E-C3: dexamethasone at 1–100 nM decreased the levels of HB and HS mRNA, whereas the decrease was reduced with higher concentrations (1 and 10 μ M, Figs. 2B–D). More importantly, dexamethasone has been shown to activate the glucocorticoid receptor and PXR with markedly different sensitivity. The glucocorticoid receptor is activated by nanomolar dexamethasone but PXR is activated by micromolar concentrations (Dostert and Heinzl, 2004; Kliewer et al., 1998). In humans, the glucocorticoid receptor has two major forms: glucocorticoid receptor- α and glucocorticoid receptor- β . The β -form is known to function as a dominant negative regulator against the α -form (Goecke and Guerrero, 2006; Dostert and Heinzl, 2004). Multiple mechanisms are proposed for the dominant negative activity including competitive DNA binding (with the α -form), formation of inactive dimers and depletion of co-activators. It appears that the co-activator depletion mechanism is better supported experimentally than the other two mechanisms (Goecke and Guerrero, 2006; Dostert and Heinzl, 2004). Throughout the paper unless otherwise specified, the glucocorticoid receptor is used to describe the α -form.

The concentration-response study was conducted with or without co-transfection of glucocorticoid receptor- β or PXR (vector was used to equalize the total amount of plasmid DNA). The results on this experiment are presented in two concentration ranges: 1–100 nM (presence or absence of glucocorticoid receptor- β , GR- β), and 0.1–100 μ M (presence or absence of PXR). As shown in Fig. 5A, the HB-4822-Luc reporter was repressed in a concentration-dependent manner with the highest repression of ~80% (100 nM). The repression, however, was significantly reduced by co-transfection of GR- β (Fig. 5A). Similarly, co-transfection of GR- β significantly reduced dexamethasone repression of the HS-1293-Luc reporter (Fig. 5B). The attenuated repression by GR- β provided further evidence that dexamethasone suppressed the expression of HB and HS through the signaling of the glucocorticoid receptor.

Increased concentrations (1–100 μ M), on the other hand, did not cause additional repression (minus-PXR, Figs. 5C and D). As a matter of fact, higher concentrations were less repressive toward the HB-4822-Luc reporter (Fig. 5C). Dexamethasone at micromolar concentrations has been shown to activate PXR (14). We next tested whether co-transfection of PXR stimulated HB and HS promoters in response to dexamethasone at high concentrations. As shown in Figs. 5C and D, both reporters were markedly activated in PXR-transfected cells. When dexamethasone was assayed at 10 μ M, for example, the HB-4822-Luc reporter was activated by ~6-fold (relative to DMSO labeled as 0) and the HS-1293-Luc by ~2 fold (Figs. 5C and D). In rats, both HB and HS were consistently suppressed regardless of single or repeated injections (Figs. 1C and D), and in cell cultures (e.g., the 24-h time-point), significant repression was detected, but the repression was reduced at high concentrations (Figs. 2C and D). The lack of net-increase in HB and HS mRNA by high concentrations or prolonged treatment suggest that rats and cultured cells, under the experimental conditions, do not express sufficient PXR. Alternatively, there is a major difference between *in vivo* and cell lines in responding to dexamethasone at high concentrations.

DISCUSSION

Carboxylesterases play an important role in xenobiotic metabolism and detoxication of insecticides (Sato and Hosokawa, 2006). The expression of several rat carboxylesterases is profoundly decreased by glucocorticoid dexamethasone (Zhu et al., 2000; Yan et al., 1995b;

Morgan et al., 1994). In this study, we have presented a molecular explanation to the suppression. Dexamethasone significantly decreased the expression of rat HA, HB and HS, and the decrease was abolished by antiglucocorticoid RU486. Additionally, dexamethasone repressed carboxylesterase promoters, and the repression was reduced by glucocorticoid receptor- β . These findings establish that dexamethasone suppresses these carboxyl-esterases through the glucocorticoid receptor.

The suppressed expression of HA, HB and HS occurred rapidly and was significant even by nanomolar dexamethasone. Such a rapid onset and large magnitude place these carboxylesterase genes as some of the most sensitive targets of dexamethasone (Becker et al., 1986; Rogatsky et al., 2002). In dexamethasone-treated rats, the levels of HA, HB and HS mRNA were decreased significantly as early as 6 h (Figs. 1B–D). Such fast decreases suggest that the transcripts of these carboxylesterases are normally decayed at a high rate in rats. In addition to the rapid onset, minimal amounts of dexamethasone evoked significant decreases. In H4-II-E-C3 cells, the mRNA levels of HB and HS were significantly decreased by 1 nM (24-h cultures) (Figs. 2C and D). In contrast, this concentration caused no significant changes in the expression of Tat (Fig. 2A), a sensitive target gene of the glucocorticoid receptor (Becker et al., 1986). The maximum suppression ranged from 60% (HA) to 80% (HB and HS) and occurred with 50 nM or lower concentrations (Figs. 2B–D).

Increased concentrations, on the other hand, were less effective on the suppression. While no net induction was detected in HB or HS mRNA by high concentrations (e.g., 10 μ M), the level of HA mRNA was significantly increased (Figs. 2B–D). A 70% increase in HA mRNA was detected with 1 μ M and a four-fold increase with 10 μ M dexamethasone in the 24-h cell cultures (Fig. 2B). The induction of HA, however, was detected in cultured cells but not whole animals (Fig. 1B). Dexamethasone-treated rats consistently expressed lower HA mRNA regardless of single or repeated injections (Fig. 1B). Apparently, the persistent suppression in rats was not due to insufficient exposure compared with the concentrations used in cell culture. It was reported that rats dosed at 0.8 mg/kg produced a C_{\max} of 682 ng/ml in the blood, equivalent to \sim 1.7 μ M (Hansen et al., 1999). Based on this estimation, a single *ip* injection of 50 mg/kg would produce a blood concentration of \sim 62.5 μ M. On the other hand, the cultured cells were exposed to dexamethasone through media whereas rats were dosed through *ip* injection. Medium exposure delivers concentrations with lesser fluctuation than *ip* injection. It is unclear whether the difference in the concentration fluctuation is a contributing factor to the difference in the regulated expression of these carboxylesterases between cultured cells and whole animals.

Interestingly, whole animals supported persistent suppression of carboxylesterases but only transient induction of TAT (Fig. 1). While the precise mechanisms remain to be determined, it is likely that rats rapidly metabolize dexamethasone and the metabolites antagonize against transactivation (Tat) but not transrepression (carboxylesterases). In support of a potential involvement of the metabolism, H4-II-E-C3 cell line was shown to sustain the induction of Tat (Fig. 2A), and cell lines usually have much lower capacity of drug-metabolism than liver (Sato and Hosokawa, 2006; Hewitt et al., 2007). Furthermore, Lamontagne et al (1984) reported that slight modifications of dexamethasone (probably mimicking metabolites) lead to increased antagonistic activity. Alternatively, the metabolites suppress the expression of carboxylesterases through a receptor rather than the glucocorticoid receptor, and this receptor shares with the glucocorticoid receptor in terms of ligand and DNA binding. Dexamethasone reportedly binds to human mineralocorticoid receptor (MR), and the signaling of MR can be reduced by glucocorticoid receptor- β as well (Goecke and Guerrero, 2006; Rebuffat et al., 2004). However, the transactivation of this receptor by dexamethasone is weak toward mammary tumor virus promoter reporter (Goecke and Guerrero, 2006; Rebuffat et al., 2004). Nevertheless, we have demonstrated in this study that anti-glucocorticoid RU486 completely

abolished dexamethasone-suppression of carboxylesterases (Fig. 3A), pointing directly to the involvement of the glucocorticoid receptor.

While the glucocorticoid receptor is involved in the suppression of the carboxylesterases by dexamethasone, it remains to be determined whether it does so through direct DNA binding, interaction with other proteins, or both. The glucocorticoid receptor reportedly exerts negative regulation through binding to a negative response element or interfering with the function of other transcription factors (Schoneveld et al., 2004). In this study, we have identified that the sequences from -620 to -559 in the HB and from -630 to -282 in the HS gene contribute significantly to the repression (Fig. 4). Interestingly, search for response elements in these regions identified no canonical motifs for this receptor, suggesting that the suppression is achieved by a non-classical binding element or interference with the functions of other transcription factor. In support of the interference mechanism, the HB-580-Luc and HB-559-Luc reporters exhibited neither responsiveness to dexamethasone nor basal activity (Fig. 4A), suggesting that dexamethasone-suppression is achieved through interfering with the function of the general transcription complex.

Nevertheless, transcriptional repression appears to be the primary mechanism that supports the suppression. Reporter assays have shown that dexamethasone repressed the carboxylesterase promoters (Fig. 4). In addition, both protein and mRNA levels were comparably altered, and the change in mRNA preceded the change of the corresponding proteins (Figs. 1B, C and E). Interestingly, compared with the decreases in the levels of mRNA and protein, the hydrolysis of *para*-nitrophenylacetate was reduced to a lesser extent (Fig. 1). For example, samples from the 96-h time-point exhibited a 60% decrease of HA and 80% decrease of HB, respectively, but the hydrolysis was decreased by only ~50% (Fig. 1E). Two possibilities likely account for such an apparent discrepancy: HA and HB contribute to only some of the hepatic hydrolysis of this ester; and dexamethasone induces a carboxylesterase that hydrolyzes *para*-nitrophenylacetate. In support of this possibility, carboxylesterase RL4, a methylprednisolone hemisuccinate hydrolase, is reportedly induced by dexamethasone (Furihata et al., 2005).

In summary, we report that dexamethasone suppresses the expression of HA, HB and HS through transcriptional repression. This steroid represses the carboxylesterase promoters, and the repression is reduced by glucocorticoid receptor- β , a dominant negative regulator of glucocorticoid receptor- α . Interestingly, the promoter sequences supporting the repression lack canonical glucocorticoid response elements, suggesting that the glucocorticoid receptor supports the suppression of rat carboxylesterases through a nonclassic mechanism. In addition, whole animals support persistent suppression regardless of single or repeated injections, whereas in cultured hepatoma cells, nanomolar concentrations cause dose-dependent suppression, but micromolar concentrations are less effective and even cause induction depending on a carboxylesterase. The liver is known to have higher metabolic capacity than cultured hepatoma cells. The difference in the metabolism, therefore, likely contributes to the difference in the responding patterns between these two systems.

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Abbreviation

AGP

	α -1 acid glycoprotein
CYP	cytochrome P450
HA	hydrolase A
HB	hydrolase B
HS	hydrolase S
MEME	minimum essential medium eagle
GAPDH	glyceradehyde-3-phosphate dehydrogenase
HCE	human carboxylesterases
PXR	pregnane X receptor
RT-qPCR	quantitative reverse transcription-polymerase chain reaction
Tat	tyrosine aminotransferase

References

- Adcock IM, Caramori G. Cross-talk between pro-inflammatory transcription factors and glucocorticoids. *Immunol Cell Biol* 2001;79:376–384. [PubMed: 11488985]
- Becker PB, Gloss B, Schmid W, Strahle U, Schutz G. In vivo protein-DNA interactions in a glucocorticoid response element require the presence of the hormone. *Nature* 1986;324:686–688. [PubMed: 2879246]
- Choudhary D, Jansson I, Sarfarazi M, Schenkman JB. Xenobiotic-metabolizing cytochromes P450 in ontogeny: evolving perspective. *Drug Metab Rev* 2004;36:549–568. [PubMed: 15554235]
- Crunkhorn SE, Plant KE, Gibson GG, Kramer K, Lyon J, Lord PG, Plant NJ. Gene expression changes in rat liver following exposure to liver growth agents: role of Kupffer cells in xenobiotic-mediated liver growth. *Biochem Pharmacol* 2004;67:107–118. [PubMed: 14667933]
- Dostert A, Heinzel T. Negative glucocorticoid receptor response elements and their role in glucocorticoid action. *Curr Pharm Des* 2004;10:2807–2816. [PubMed: 15379669]
- Furihata T, Hosokawa M, Fujii A, Derbel M, Satoh T, Chiba K. Dexamethasone-induced methylprednisolone hemisuccinate hydrolase: its identification as a member of the rat carboxylesterase 2 family and its unique existence in plasma. *Biochem Pharmacol* 2005;69:1287–1297. [PubMed: 15794950]
- Goecke A, Guerrero J. Glucocorticoid receptor beta in acute and chronic inflammatory conditions: clinical implications. *Immunobiology* 2006;211:85–96. [PubMed: 16446173]
- Hansen DK, LaBorde JB, Wall KS, Holson RR, Young JF. Pharmacokinetic considerations of dexamethasone-induced developmental toxicity in rats. *Toxicol Sci* 1999;48:230–239. [PubMed: 10353314]
- Hewitt NJ, Lechon MJ, Houston JB, Hallifax D, Brown HS, Maurel P, Kenna JG, Gustavsson L, Lohmann C, Skonberg C, Guillouzo A, Tuschl G, Li AP, LeCluyse E, Groothuis GM, Hengstler JG. Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins,

- and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. *Drug Metab Rev* 2007;39:159–234. [PubMed: 17364884]
- Klein ES, DiLorenzo D, Posseckert G, Beato M, Ringold GM. Sequences downstream of the glucocorticoid regulatory element mediate cycloheximide inhibition of steroid induced expression from the rat alpha 1-acid glycoprotein promoter: evidence for a labile transcription factor. *Mol Endocrinol* 1988;2:1343–1351. [PubMed: 3216868]
- Kliwer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstrom RH, Perlmann T, Lehmann JM. An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* 1998;92:73–82. [PubMed: 9489701]
- Kroetz DL, McBride OW, Gonzalez FJ. Glycosylation-dependent activity of baculovirus-expressed human liver carboxylesterases: cDNA cloning and characterization of two highly similar enzyme forms. *Biochemistry* 1993;32:11606–11617. [PubMed: 8218228]
- Lamontagne N, Mercier L, Pons M, Thompson EB, Simons SS Jr. Glucocorticoid versus antigluco-corticoid activity: can a single functional group modification of glucocorticoid steroids always convey antigluco-corticoid activity? *Endocrinology* 1984;114:2252–2263. [PubMed: 6547091]
- Morgan EW, Yan B, Greenway D, Parkinson A. Regulation of two rat liver microsomal carboxylesterase isozymes: species differences, tissue distribution and the effects of age, sex and xenobiotic treatment of rats. *Arch Biochem Biophys* 1994;315:514–526.
- Onvic M, Swank RT, Fletcher C, Zhen L, Novak EK, Baumann H, Heintz N, Ganschow RE. Characterization and functional expression of a cDNA encoding egasyn esterases-22: the endoplasmic reticulum-targeting proteins of β -glucuronidase. *Genomics* 1991;11:956–967. [PubMed: 1783403]
- Pei L. Identification of a negative glucocorticoid response element in the rat type 1 vasoactive intestinal polypeptide receptor gene. *J Biol Chem* 1996;271:20879–20884. [PubMed: 8702844]
- Poole M, Bridgers K, Alexson SE, Corton JC. Altered expression of the carboxylesterases ES-4 and ES-10 by peroxisome proliferator chemicals. *Toxicology* 2001;165:109–19. [PubMed: 11522369]
- Rebuffat AG, Tam S, Nawrocki AR, Baker ME, Frey BM, Frey FJ, Odermatt A. The 11-ketosteroid 11-ketodexamethasone is a glucocorticoid receptor agonist. *Mol Cell Endocrinol* 2004;214:27–37. [PubMed: 15062542]
- Rogatsky I, Luecke HF, Leitman DC, Yamamoto KR. Alternate surfaces of transcriptional coregulator GRIP1 function in different glucocorticoid receptor activation and repression contexts. *Proc Natl Acad Sci U S A* 2002;99:16701–16706. [PubMed: 12481024]
- Satoh T, Hosokawa M. Structure, function and regulation of carboxylesterases. *Chem Biol Interact* 2006;162:195–211. [PubMed: 16919614]
- Satoh T, Suzuki S, Kawai N, Nakamura T, Hosokawa M. Toxicological significance in the cleavage of esterase-beta-glucuronidase complex in liver microsomes by organophosphorus compounds. *Chem Biol Interact* 1999;119–120:471–478. [PubMed: 10421485]
- Schoneveld OJ, Gaemers IC, Lamers WH. Mechanisms of glucocorticoid signalling. *Biochim Biophys Acta* 2004;1680:114–128. [PubMed: 15488991]
- Schwer H, Langmann T, Daig R, Becker A, Aslanidis C, Schmitz G. Molecular cloning and characterization of a novel putative carboxylesterase, present in human intestine and liver. *Biochem Biophys Res Commun* 1997;233:117–120. [PubMed: 9144407]
- Shi D, Yang J, Yang D, LeCluyse EL, Black C, You L, Akhlaghi F, Yan B. Anti-influenza prodrug oseltamivir is activated by carboxylesterase human carboxylesterase 1, and the activation is inhibited by antiplatelet agent clopidogrel. *J Pharmacol Exp Ther* 2006;319:1477–1484. [PubMed: 16966469]
- Tang M, Mukundan M, Yang J, Charpentier N, LeCluyse EL, Black C, Yang D, Shi D, Yan B. Antiplatelet agents aspirin and clopidogrel are hydrolyzed by distinct carboxylesterases, and clopidogrel is transesterified in the presence of ethyl alcohol. *J Pharmacol Exp Ther* 2006;319:1467–1476. [PubMed: 16943252]
- Yan B, Yang D, Brady M, Parkinson A. Rat kidney carboxylesterase: cloning, sequencing, cellular localization and relationship to liver hydrolase B. *J Biol Chem* 1994;269:29688–29696. [PubMed: 7961958]

- Yan B, Yang D, Brady M, Parkinson A. Rat testicular carboxylesterase: cloning, sequencing, cellular localization and relationship to liver hydrolase A. *Arch Biochem Biophys* 1995a;316:899–908. [PubMed: 7864649]
- Yan B, Yang D, Bullock P, Parkinson A. Rat serum carboxylesterase. cloning, expression, regulation, and evidence of secretion from liver. *J Biol Chem* 1995b;270:19128–19134. [PubMed: 7642579]
- Yang J, Shi D, Yang D, Song X, Yan B. Interleukin-6 suppresses the expression of carboxyl-esterases HCE1 and HCE2 through transcriptional repression. *Mol Pharmacol* 2007;72:686–694. [PubMed: 17537833]
- Yang J, Liao M, Shou M, Jamei M, Yeo KR, Tucker GT, Rostami-Hodjegan A. Cytochrome p450 turnover: regulation of synthesis and degradation, methods for determining rates, and implications for the prediction of drug interactions. *Curr Drug Metab* 2008;9:384–394. [PubMed: 18537575]
- Zhang H, LeCluyse E, Liu L, Hu M, Matoney L, Yan B. Rat pregnane X receptor: molecular cloning, tissue distribution and xenobiotic regulation. *Arch Biochem Biophys* 1999;368:14–22. [PubMed: 10415106]
- Zhang X, Clark AF, Yorio T. Regulation of glucocorticoid responsiveness in glaucomatous trabecular meshwork cells by glucocorticoid receptor-beta. *Invest Ophthalmol Vis Sci* 2005;46:4607–4616. [PubMed: 16303956]
- Zhu W, Song L, Zhang H, Matoney L, LeCluyse E, Yan B. Dexamethasone differentially regulates expression of carboxylesterase genes in humans and rats. *Drug Metab Dispos* 2000;28:186–191. [PubMed: 10640517]

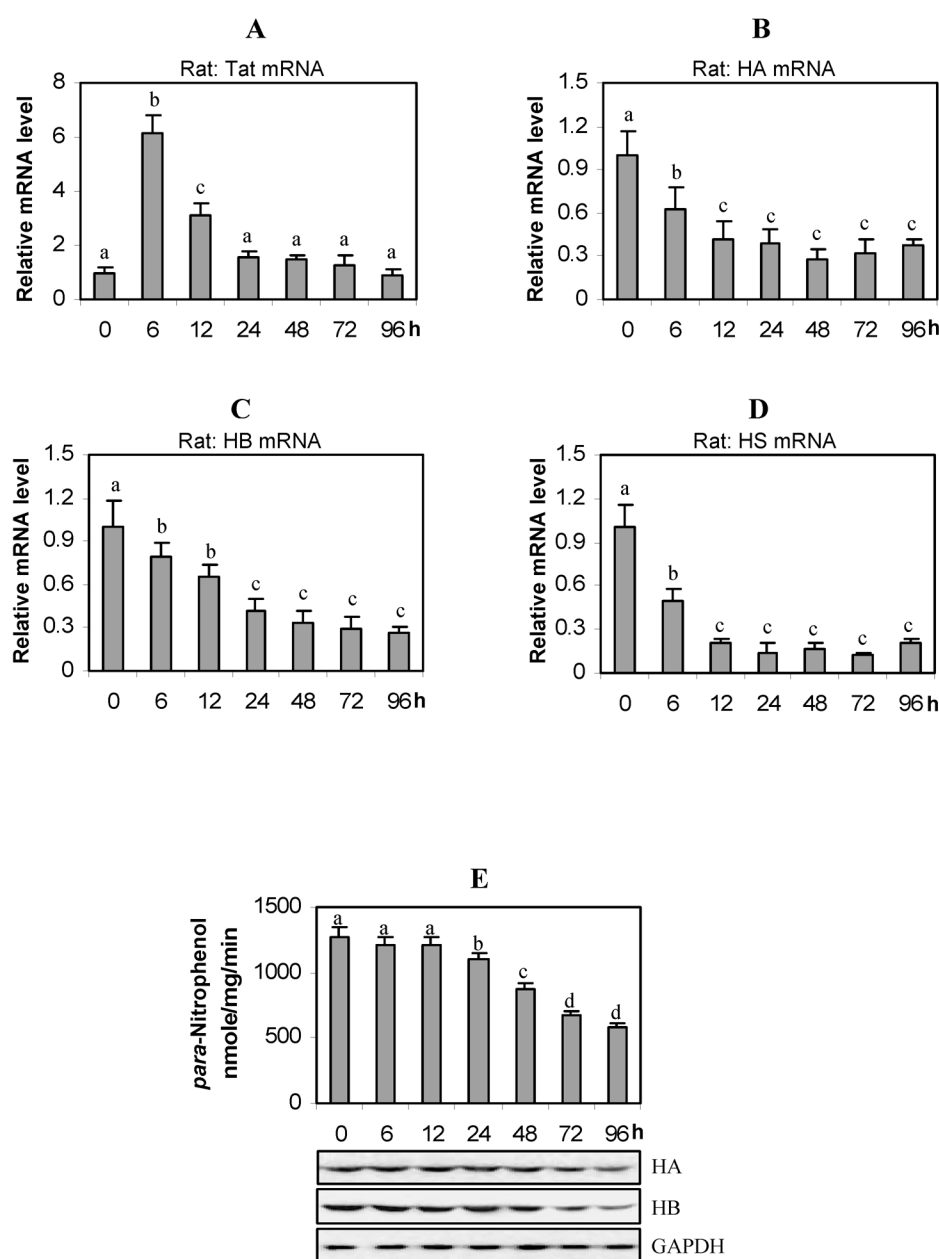


Fig. 1. Effect of dexamethasone on the expression of Tat, HA, HB and HS *in vivo*

Male rats (8-week old, 3 per group) were treated with dexamethasone at 50 mg/kg for the maximum of four consecutive days and sacrificed at 6, 12, 24, 48, 72 and 96 h after the initial treatment. Rats sacrificed before or at 24 h received only a single injection. The livers were perfused with saline through the portal vein to remove the blood. Total RNA and S9 fractions were prepared and analyzed for the expression of Tat, HA, HB and HS by RT-qPCR and Western blotting. The S9 fractions were also analyzed for the hydrolysis of *para*-nitrophenylacetate. The mRNA levels were normalized and expressed as ratios over those from the corresponding controls. **(A) RT-qPCR analysis of Tat** The Ct values (threshold cycles) for controls are: Tat (0 h), 20.14 ± 0.147 (mean \pm SD); Tat (6 h), 20.37 ± 0.212 ; Tat (12 h), 20.21 ± 0.240 ; Tat (24 h), 19.95 ± 0.234 ; Tat (48 h), 19.87 ± 0.239 ; Tat (72 h), 19.80 ± 0.317 and Tat (96 h), 19.74 ± 0.237 . **(B) RT-qPCR analysis of HA** The Ct values for controls are:

HA (0 h), 19.91 ± 0.339 ; HA (6 h), 19.97 ± 0.235 ; HA (12 h), 20.25 ± 0.275 ; HA (24 h), 20.72 ± 0.344 ; HA (48 h), 20.39 ± 0.309 ; HA (72 h), 20.21 ± 0.286 and HA (96 h), 20.43 ± 0.288 .

(C) RT-qPCR analysis of HB The Ct values for controls are: HB (0 h), 21.43 ± 0.332 ; HB (6 h), 21.34 ± 0.290 ; HB (12 h), 21.70 ± 0.346 ; HB (24 h), 21.56 ± 0.289 ; HB (48 h), 21.89 ± 0.252 ; HB (72 h), 21.94 ± 0.566 and HB (96 h), 21.54 ± 0.340 .

(D) RT-qPCR analysis of HS The Ct values for controls are: HS (0 h), 19.84 ± 0.263 ; HS (6 h), 19.87 ± 0.225 ; HS (12 h), 19.75 ± 0.315 ; HS (24 h), 19.65 ± 0.378 ; HS (48 h), 20.05 ± 0.169 ; HS (72 h), 20.13 ± 0.538 and HS (96 h), 20.01 ± 0.429 . **(E) Effect of dexamethasone treatment on the hydrolysis of *para*-nitrophenylacetate and expression of HA, HB** Hydrolysis of *para*-nitrophenylacetate was determined spectrophotometrically with 20 μ g protein and 1 mM substrate and the levels of HA and HB were determined by Western analysis (4 μ g for HA and 8 μ g for HB) as described in Materials and Methods. Bars with a different letter indicate statistically significant differences ($p < 0.05$).

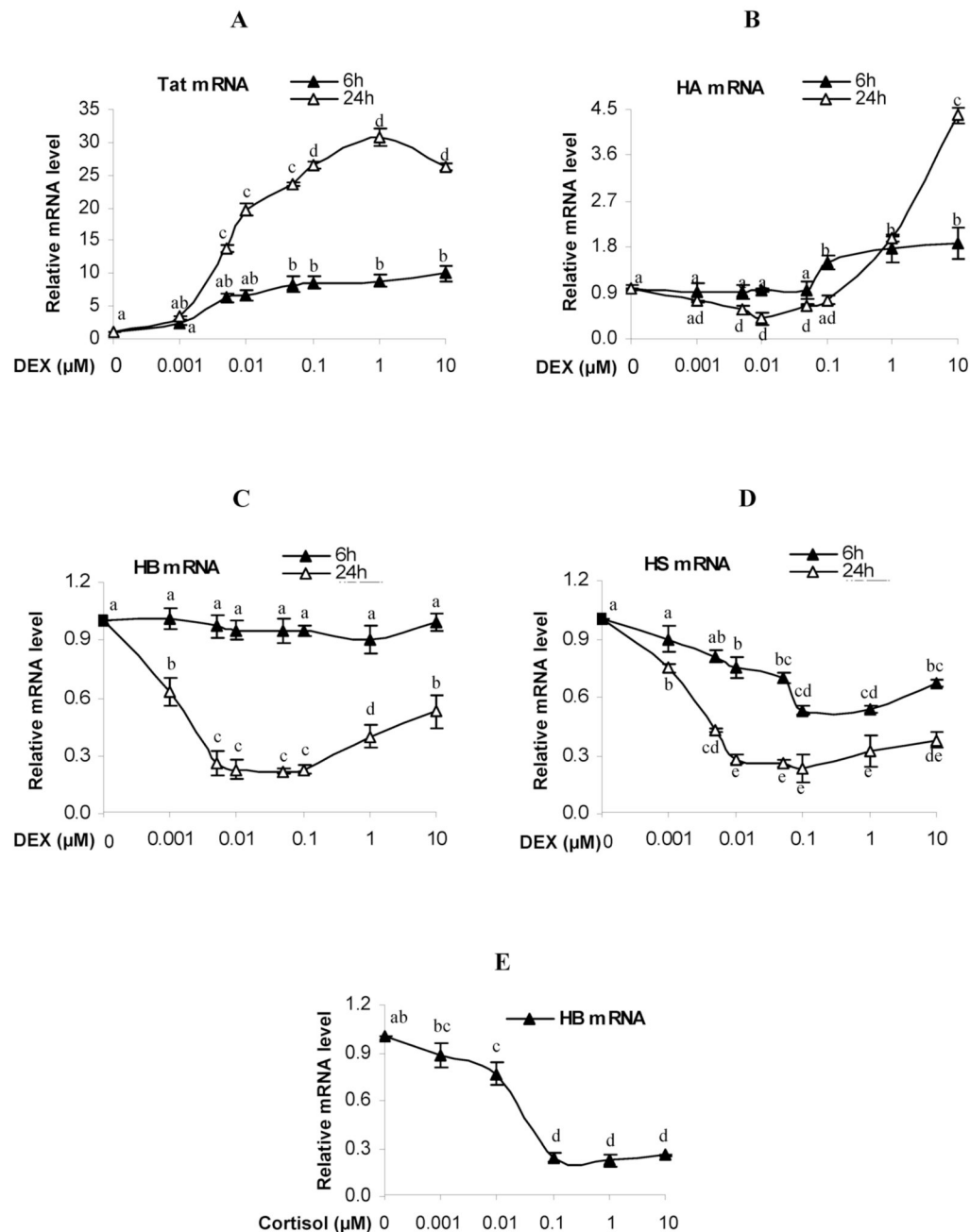


Fig. 2. Effect of dexamethasone or cortisol on the level of Tat, HA, HB or HS mRNA in hepatoma cell line

H4-II-E-C3 cells were seeded at a density of 5×10^5 in 12-well plates. After an overnight incubation, the cells were treated with dexamethasone at various concentrations (0–10 μ M) for 6–24 h. Total RNA was isolated and analyzed for the level of Tat, HA, HB and HS mRNA by RT-qPCR. (A) **RT-qPCR analysis of Tat** The Ct values for controls are: Tat (6 h), 21.82 ± 0.153 and Tat (24 h), 21.60 ± 0.265 . (B) **RT-qPCR analysis of HA** The Ct values for controls are: HA (6 h), 31.62 ± 0.394 and HA (24 h), 31.95 ± 0.513 . (C) **RT-qPCR analysis of HB** The Ct values for controls are: HB (6 h), 22.49 ± 0.103 and HB (24 h), 22.26 ± 0.333 . (D) **RT-qPCR analysis of HS** The Ct values for controls are: HS (6 h), 26.01 ± 0.460 and HS (24 h), 25.86 ± 0.146 . (E) **Effect of cortisol on the level of HB mRNA** H4-II-E-C3 cells were cultured

as described above and treated with cortisol at various concentrations (0–10 μ M) for 24 h. The level of HB mRNA was monitored by RT-qPCR. The Ct value for the control is: HB (24 h), 22.56 ± 0.121 . Data presented in this figure were assembled from three independent experiments. A different letter between two values indicates statistically significant differences ($p < 0.05$).

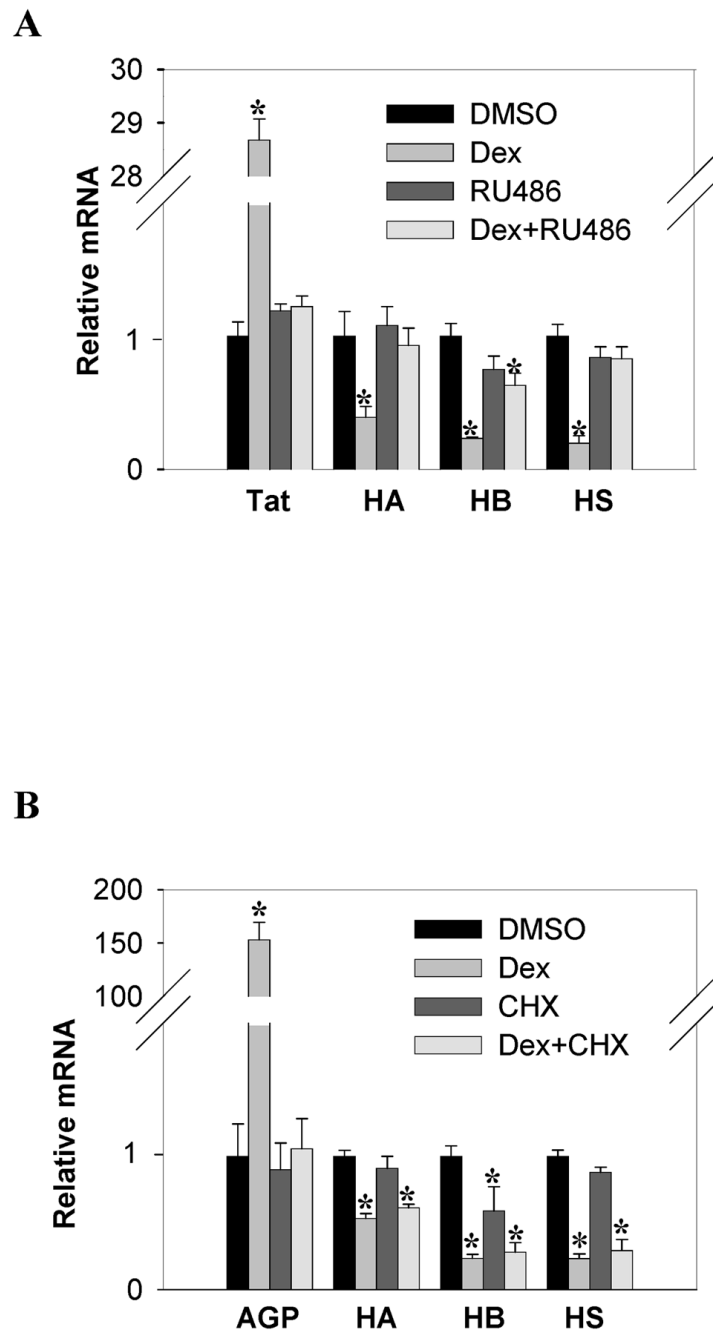
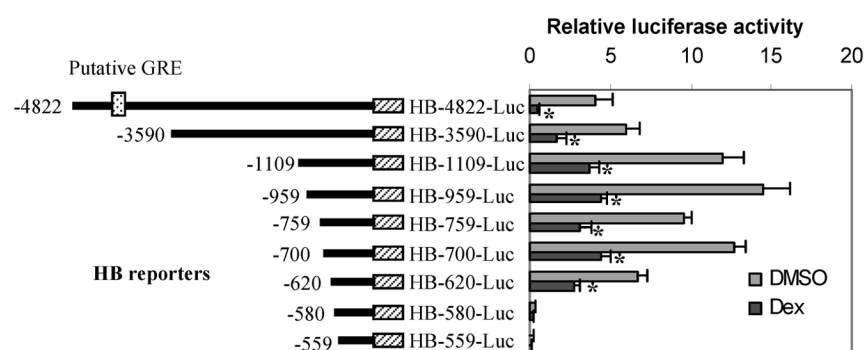


Fig. 3. Effect of RU486 or cycloheximide on the dexamethasone-regulated expression of HA, HB and HS (A) Effect of RU486 on the dexamethasone-regulated expression of Tat, HA, HB and HS H4-II-E-C3 cells were seeded at a density of 5×10^5 in 12-well plates. After an overnight incubation, the cells were treated with dexamethasone (Dex: 50 nM for HB and HS but 10 nM for HA), RU486 (50 nM) or both for 24 h. The level of Tat, HA, HB and HS mRNA was determined by RT-qPCR. The Ct values for controls are: Tat, 21.58 ± 0.293 ; HA, 31.48 ± 0.327 ; HB, 22.24 ± 0.280 and HS, 25.78 ± 0.226 . **(B) Effect of cycloheximide on the dexamethasone-regulated expression of AGP, HA, HB and HS** H4-II-E-C3 cells were cultured as above. The cells were treated with dexamethasone (Dex: 50 nM for HB and HS but 10 nM for HA), cycloheximide (CHX, 1 μ M) or both for 24 h. The level of AGP, HA, HB and

HS mRNA was determined. The mRNA levels were normalized and expressed as ratios over those from the corresponding controls. Data presented in this figure were assembled from three independent experiments. *Statistically significant difference from the vehicle control ($p < 0.05$). The Ct values for controls are: AGP, 34.68 ± 0.445 ; HA, 31.90 ± 0.147 ; HB, 22.11 ± 0.276 and HS, 25.75 ± 0.180 .

A



B

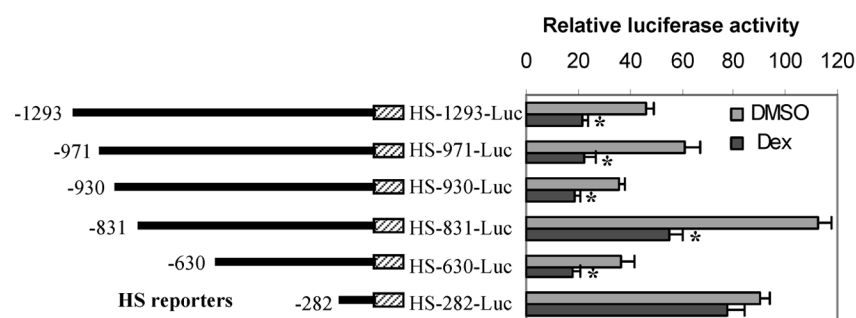


Fig. 4. Repression of HB and HS promoter reporters by dexamethasone

H4-II-E-C3 cells were seeded in 48-well plates at a density of 1.2×10^5 . After an overnight incubation, the cells were transiently transfected by FuGene HD with a mixture containing a reporter (100 ng) along with 10 ng of the tk-Renilla luciferase plasmid. After incubation at 37°C for 24 h (serum-free medium), the transfected cells were treated with dexamethasone (50 nM) or the same volume of DMSO for 48 h. Luciferase activities were determined with a Dual-Luciferase Reporter Assay System and the reporter activity was normalized based on the Renilla luminescence signal. (A) **HB reporters**, and (B) **HS reporters**. Data presented in this figure were assembled from three independent experiments and each experiment was performed in triplicate. *Statistically significant difference from the vehicle control for each reporter ($p \leq 0.05$).

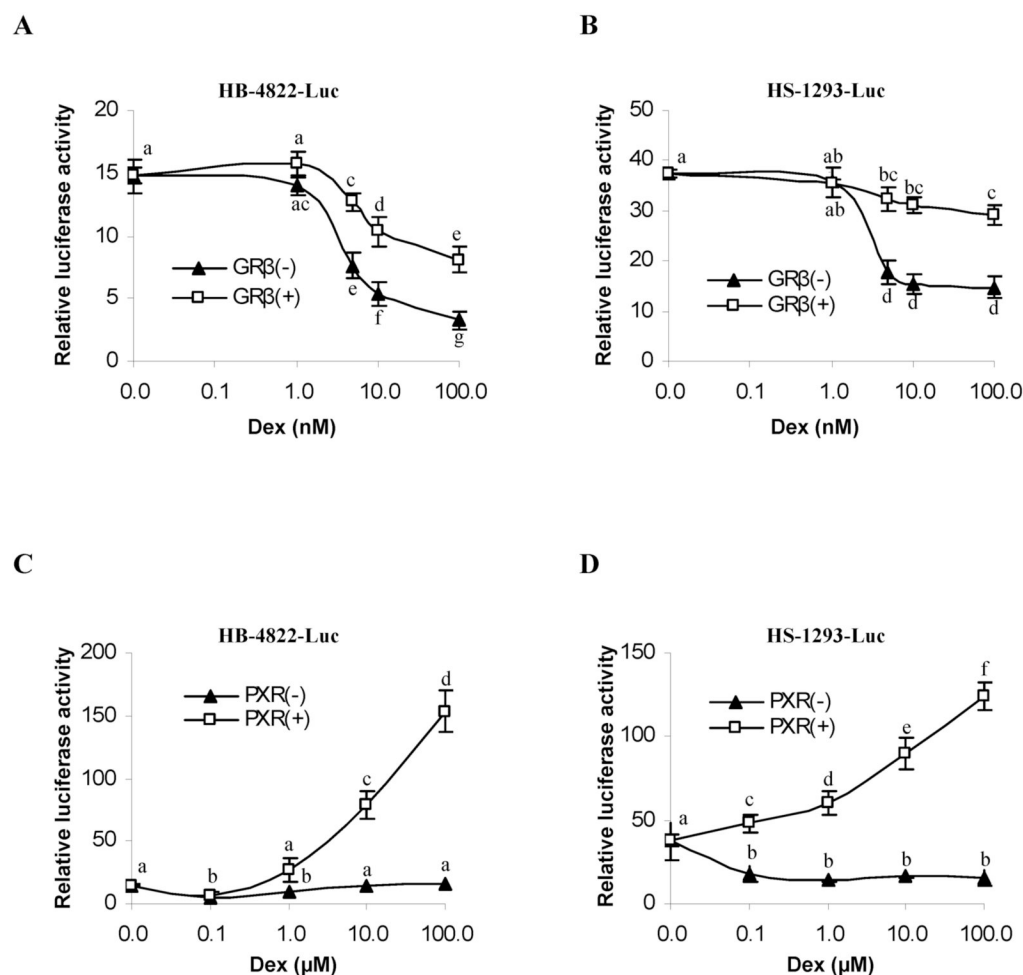


Fig. 5. Effect of co-transfected glucocorticoid receptor-β or PXR on dexamethasone-repression of HB and HS promoters

H4-II-E-C3 cells were seeded in 48-well plates at a density of 1.2×10^5 . After an overnight incubation, the cells were transiently transfected by FuGene HD with a mixture containing a reporter (100 ng) and the tk-Renilla luciferase plasmid (10 ng) along with glucocorticoid receptor-β (GR-β), PXR or vector (100 ng). After incubation at 37°C for 24 h (serum-free medium), the transfected cells were treated with dexamethasone (50 nM) or the same volume of DMSO for 48 h. Luciferase activities were determined with a Dual-Luciferase Reporter Assay System and the reporter activity was normalized based on the Renilla luminescence signal. (A) Repression of the HB-4822-Luc reporter in cells co-transfected with GR-β or vector; (B) Repression of the HS-1293-Luc reporter in cells co-transfected with GR-β or vector; (C) Repression of the HB-4822-Luc reporter in cells co-transfected with PXR or vector; and (D) Repression of the HS-1293-Luc reporter in cells co-transfected with PXR or vector. Data presented in this figure were assembled from three independent experiments and each experiment was performed in triplicate. Data-points with a different letter (sharing no letter) indicate statistically significant differences ($p < 0.05$).

Table I

Sequences of primers for reporter constructs

Primer	Sequence	Reporter
HB-3590XhoIs	5'-ccttatctcgagtcgttgaaactgacaatgttactat-3'	HB-3590-Luc
HB-1109XhoIs	5'-atactactcgaggtagaggcattttggatcac-3'	HB-1109-Luc
HB-959XhoIs	5'-atactactcgagtcacagctcccttgcgtgagg-3'	HB-959-Luc
HB-759XhoIs	5'-atactactcgagctgtcctcacatctggtctgg-3'	HB-759-Luc
HB-700XhoIs	5'-atactactcgagtggtaaaaagcaagtgtttt-3'	HB-700-Luc
HB-620XhoIs	5'-atactactcgagtggttccaagaggggtca-3'	HB-620-Luc
HB-580XhoIs	5'-atactactcgagcttgcaagactgttatatg-3'	HB-580-Luc
HB-559XhoIs	5'-atactactcgagactaatctagaattctccaag-3'	HB-559-Luc
HB-172BglIIa	5'-aagccagatctctccaagtttaacctgctttatct-3'	all HB reporters
HB-4822KpnIs	5'-tgtcaaggtaccgatatatgctctctcctgtttc-3'	HB-4822-Luc
HB-3537NheIa	5'-tagggtagctcccaactggcagacttatccaaga-3'	HB-3537-Luc
HS-1293XhoIs	5'-tctccctcgaggtacattccaatggcttaatgccga-3'	HS-1293-Luc
HS-971XhoIs	5'-catagctcgagaaatgtgtggatgattagacaaag-3'	HS-971-Luc
HS-930XhoIs	5'-ggttactcgagttttatttgcataagaacaaagt-3'	HS-930-Luc
HS-831XhoIs	5'-gtgctgctcgaggatatttatgagatgtagcct-3'	HS-831-Luc
HS-630XhoIs	5'-ctcttctcgaggttgcccttgcctctctgtttcatc-3'	HS-630-Luc
HS-282XhoIs	5'-cctgtctcgagaagttcaaggtgcactgagaatttcg-3'	HS-282-Luc
HS-16EcoRVa	5'-atctcttgagacagggaggtgtctactgctcaaat-3'	all HS reporters